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**IMMUNOGENICITY AND INTEGRATION OF A DECELLULARIZED
EXTRACELLULAR-MATRIX SCAFFOLD FOR THE RECONSTRUCTION OF
HUMAN FORESKIN: EXPERIMENTAL STUDY IN ANIMAL MODEL**

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ABSTRACT

Circumcision is one of the most surgical techniques performed around the world just considered that globally about 38% of people and 2.6% in Italy are circumcised.

Million of people suffer from physical and psychological damage caused by circumcision. To date, there is not yet therapeutic and effective surgery for foreskin restoration. Recently, a group of Italian researchers have developed a novel decellularized method for epithelial tissue to obtain an extracellular matrix scaffold derived from human donor foreskin.

The aim of this study was to evaluate the immune response and integration of this scaffold when implanted in the host.

The study, approved by Ministry of Health (n. 424/2021-PR), involved twenty-six Wistar male rats. Under complete asepsis, an infrascapular skin incision of about 1 cm was made and a decellularized membrane (about 2 cm of diameter) was implanted in hypodermal layer. To evaluate different stages of inflammatory response, rats were subdivided in two groups: A group (13 rats) that had a follow up of 30 days; B group (13 rats) that had a follow up only of 5 days. The infrascapular region was monitored in attempt to score key signs of inflammation: calor, rubor and tumor, scoring from 0 (no alterations) to 3 (severe alterations). After 5 days for B group and 30 days for A group, the subjects underwent general anesthesia and euthanasia. A tissue explant centered on the surgical scar was performed and sent to the pathology lab for histological and immunohistochemical analyses, in attempt to assess the presence and degree of the inflammatory infiltrate, related to the host's immune response to the implant.

The clinical evaluation showed slight signs of inflammation in the first five days post implantation. After this period, all clinical scores were 0 for the remaining 25 days.

Histological and immunohistochemical finds highlighted a mild acute inflammatory response in both groups at the two different times of the study. Differently, a moderate degree of chronic inflammation was observed.

The analysis of data related to scaffold integration showed interesting results. Specifically, neovascularization and cell colonization were significantly higher in A compared to B group. In contrast, the presence of the capsule was mild in all subjects, and it was no different between two groups of the study.

In conclusion, the decellularized extracellular matrix derived from human foreskin did not evoke a significative immune response and supported the neovascularization and cell colonization when implanted in the host.

1. INTRODUCTION

Every year, about 13.3 million of boys are circumcised in the world and about 10.000 in Italy. It has been reported that the circumcision adversely affects sexual function in many men and results psychological adverse in infants.

To date, there is not a treatment protocol for definitive foreskin restoration due to its complex structure rich in vascularization and densely innervated.

In this context, it was born Foregen Onlus, a non-profit organization founded to research and improve regenerative strategies for the foreskin reconstruction for circumcised males.

In the 2018, the Emilia Romagna Regional Skin Bank in collaboration with Foregen Onlus were developed a new promising decellularized extracellular-matrix scaffold for the reconstruction of human foreskin.

Unlike biological and synthetic biomaterial matrices, this decellularized extracellular matrix is characterized to the presence of the same intrinsic anatomic and structural components that are biologically inherent in the foreskin tissue.

However, this scaffold needs to in vivo studies to evaluate the real feasibility and efficacy for foreskin regeneration.

These were the condition for beginning the collaboration between Foregen and the University of Camerino.

The goal was to evaluate the proprieties of decellularized scaffold produced by the Emilia Romagna Regional Skin Bank in animal model.

Particularly, it was used in murine model and successively in the ovine model.

Lastly, the outcomes achieve in animal model will be translated in human patient.

In this thesis will be discussed in detail the host response when the scaffold is implanted in rat model.

In ovine model, it will be developed a microsurgical technique for the promotion of the engraftment and revascularization of decellularized foreskin.

The outcomes obtained in rat model will be used as a catalyst for the ovine and human studies.

2.1. ANATOMY AND FUNCTION OF SKIN

The skin is the largest organ of body. It is involved in several function: protection against mechanical, thermal, biologic and chemical agents, vitamin D3 synthesis, thermoregulation, excretion, water retention and immune response (Kolarsick et al., 2011).

Under the skin, there are fat cells (adipocytes) that make up the subcutis or hypodermis. The skin, the hypodermis and skin appendages form the integumentary system (Driskell et al., 2014).

The skin is composed of two layers: epidermidis and dermis. The epidermidis is the surface layer formed from ectoderm and it represents about 5% of the skin. The dermis is the deep layer formed from mesoderm. It supplies the epidermidis with nutrient and represents about 95% of the skin (Barone and Paul, 2012; Konig and Liebich, 2014).

The epidermidis is a stratified squamous epithelium. It is composed of five main layers:

- Stratum corneum: is uppermost layer and is composed of keratinocytes which are eliminated through sloughing.
- Stratum lucidum: consists of one or two layers of keratinocytes that contain a translucent substance.
- Stratum granulosum: is a relatively thin layer, in which the composition and shape of keratinocytes significantly change.
- Stratum spinosum: consists of four-ten layer of keratinocytes which modify themselves into polygonal shapes.
- Stratum germinativum also called basal: is the deepest and adheres to the underlying dermis. It consists of one cubiform keratinocytes layer. In addition, there are also melanocytes and a small number of Merkel cells (Ballarin and Radaelli, 2012; Yousef et al, 2014).

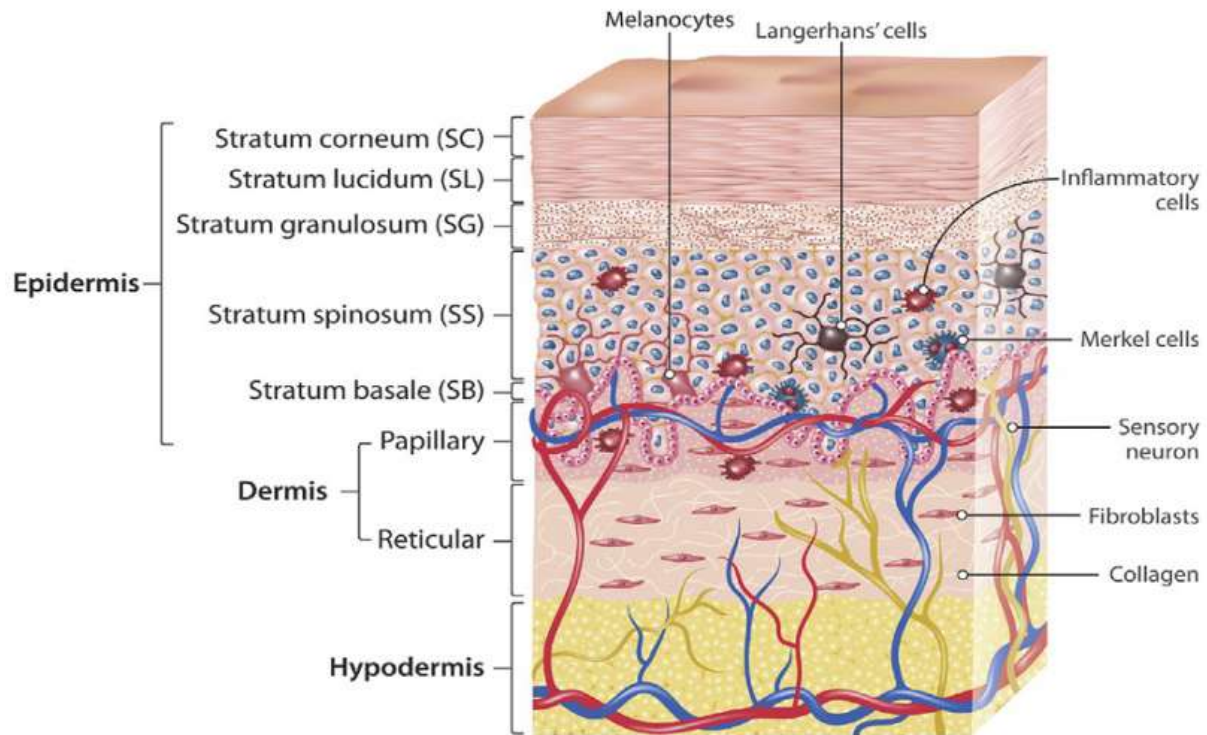


Figure 1. The multilayer structure of human skin tissue (Rahmati et al., 2020).

As the predominant cell type, the keratinocytes constitute 85% of the cellular population found in the epidermis. The stratified squamous epithelium resists abrasion because the loss of superficial cells does not affect the underlying tissue. It is composed of cells characterized by tight junction. The basal layer lies on the basal membrane through hemidesmosomes of column-shaped cells providing a strength attachment between epidermis and dermis. It consists of stem cells that divide by mitosis and differentiate in several layers, up until the lucidum and corneum layers. This last is formed by corneocytes that are flattened keratinocytes in their last stage of differentiation. The basal layer cells are also responsible for the signal transduction that modulate the architecture of cytoskeleton, cell growth and differentiation (McGavin and Zachary, 2019; König and Liebich, 2014.; Walko et al., 2015)

The apical surface of basal layer is connected to stratum spinosum. This layer presents polygonal cells with active protein synthesis. The polygonal cells produce large number of cytokeratin (intermediate filament proteins) that form the desmosomes. The desmosomes constitute a filament network of adjacent keratinocytes, ensuring the epithelium has high resistance. They give layer the spine-like aspect. In the spinosum layer, the keratinocytes mature and flatten to form the stratum granulosum. These cells synthesize keratoin dense

granules rich in proteins such as involucrin and locrin (Kolarsick et al., 2011; Walko et al, 2015; Freeman and Sonthalia, 2023).

The stratum corneum consist of scales that are self-engulfment keratinocytes without nucleus and cytoplasm. These flat and interdigitating cells are usually dead. The stratum corneum acts as the skin barrier that protects against mechanical insults, and excessive loss of water from exiting body. In addition to scales, lipids resulting from membrane cells degradation and sebaceous gland secretion play a role in supporting the skin barrier (Anderton and Alqudah, 2022).

Melanocytes, Langerhans cells, and Merkel cells are also present in epidermidis, especially concentrated in basal layer, although more incidentally (15%) (de Szalay and Wertz, 2023).

Melanocytes are dendritic cells formed from neuronal crest and produce melanin. They contain intracytoplasmic organelle known as the melanosome in which the melanin is produced. Melanosomes, via tyrosinases enzymes, convert tyrosine into eumelanin and pheomelanin. Melanocytes transfer melanin to keratinocytes based on ultraviolet radiation and autocrine and paracrine factors. This process is called “pigmentation” and represent an important defense mechanism for the epidermidis (Cichorek et al., 2013).

Langerhans cells are dendritic cells derived from bone marrow. They represent 3-4% of epidermidis cells and contain cones or rack shape organelles known as Bierbeck granules. Langerhans cells are involved in immune and allergic responses of the skin (Jaitley and Saraswathi, 2012).

Merkel cells are oval shape cells and provide receptor function to mechanical stimuli. They are the simplest touch sensors and register the pressure on the skin (Bataille et al., 2022).

The epidermis is separated from dermis by the dermal-epidermal junction called basal membrane. This is permeable to oxygen and nutrients from dermis (Kolarsick et al. 2011; Aleemardani et al., 2021).

It is composed of three main structures:

- Lamina lucida: is connected to keratinocytes of basal layer by the anchoring filaments of hemidesmosomes.
- Basal lamina: is a structure of extracellular matrix produced by the epithelial cells.
- Anchoring fibrils: are composed prevalently of type VII collagen (Aleemardani et al., 2021)

The dermis, or chorion, derives from mesenchyme and nourishes, and supports epidermis. Its structure consists of collagen, elastic fiber, and extrafibrillar matrix. The dermis is rich in blood vessels and nerve endings. It supports hair follicles, sweat glands, sebaceous glands (oil glands), apocrine glands, lymphatic vessels, nerves, and blood vessels. Fibroblasts, macrophages, and mast cells are three major types of cells in dermis. It is formed by two layers: papillary and reticular dermis (Brown and Krishnamurth, 2022; Wong et al., 2016)

The papillary dermis is the superficial layer. It is formed by loose connective tissue and contains a lot of collagen fibers. The papillary dermis present dermal papillae that are interdigitations of dermis with the function to increase the contact surface of epidermis and to promote the nutrients diffusion. They are characteristic of surfaces subjected to mechanical stimulation such as paw pads of carnivores or equine hoof (Kanitakis, 2002).

The reticular dermis is the lower layer. It consists of abundant and robust collagen and elastic fibers. This layer supports the base of the hair follicle and the apocrine glands (Brown and Krishnamurthy, 2022).

The hypodermis or subcutis connects the skin to underlying tissues. It consists prevalently of loose connective tissue. Adipose cells, fibroblasts and macrophages are present in the subcutis. In some anatomy region, the hypodermis is rich in adipose cells, and it is called panniculus adiposus. This last is an energy reserve and acts as a thermal insulation (Driskell et al., 2014)

Three connective tissue layers are identified in hypodermis: superficial lamina (loose connective tissue), intermediate and deep lamina (both dense connective tissue) (Driskell et al., 2014).

The integumentary system has an abundant vascularization that supports the skin structures and involves in body thermoregulation (Mauldin and Peters-Kennedy, 2016)

This vascularization is constituted of three different plexuses: the superficial or subpapillary plexus, intermediate plexus, and deep plexus or subcutis plexus (at hypodermis level). The superficial plexus supplies the surface of the hair follicles and the epidermis. It is a ramification of ascendent arterioles at the junction between the papillary and reticular dermis. The intermediate plexus supplies the arrector pili muscle, the intermediate portion of hair follicles and the sebaceous glands at dermal-epidermal junction. The deep plexus supplies the base of hair follicles and the apocrine glands. Each deep artery of subcutis plexus physiologically supplies a circumscribed anatomical region and the anastomoses of its to nearby arteries are closed. These anastomoses are activated following local compression or occlusion afferent arteries (Barone and Paul, 2012; Braverman, 2000).

The venous system takes the same course of the artery. The arteriovenous anastomoses allow much of heat to be lost from the body. The epidermidis does not contain lymphatic and blood vessels. The lymphatic system consists of two networks (superficial and deep): the superficial running across the dermal papillae near the subpapillary plexus that drains into deep plexus (McGavin and Zachary, 2019; Imanishi et al., 2008).

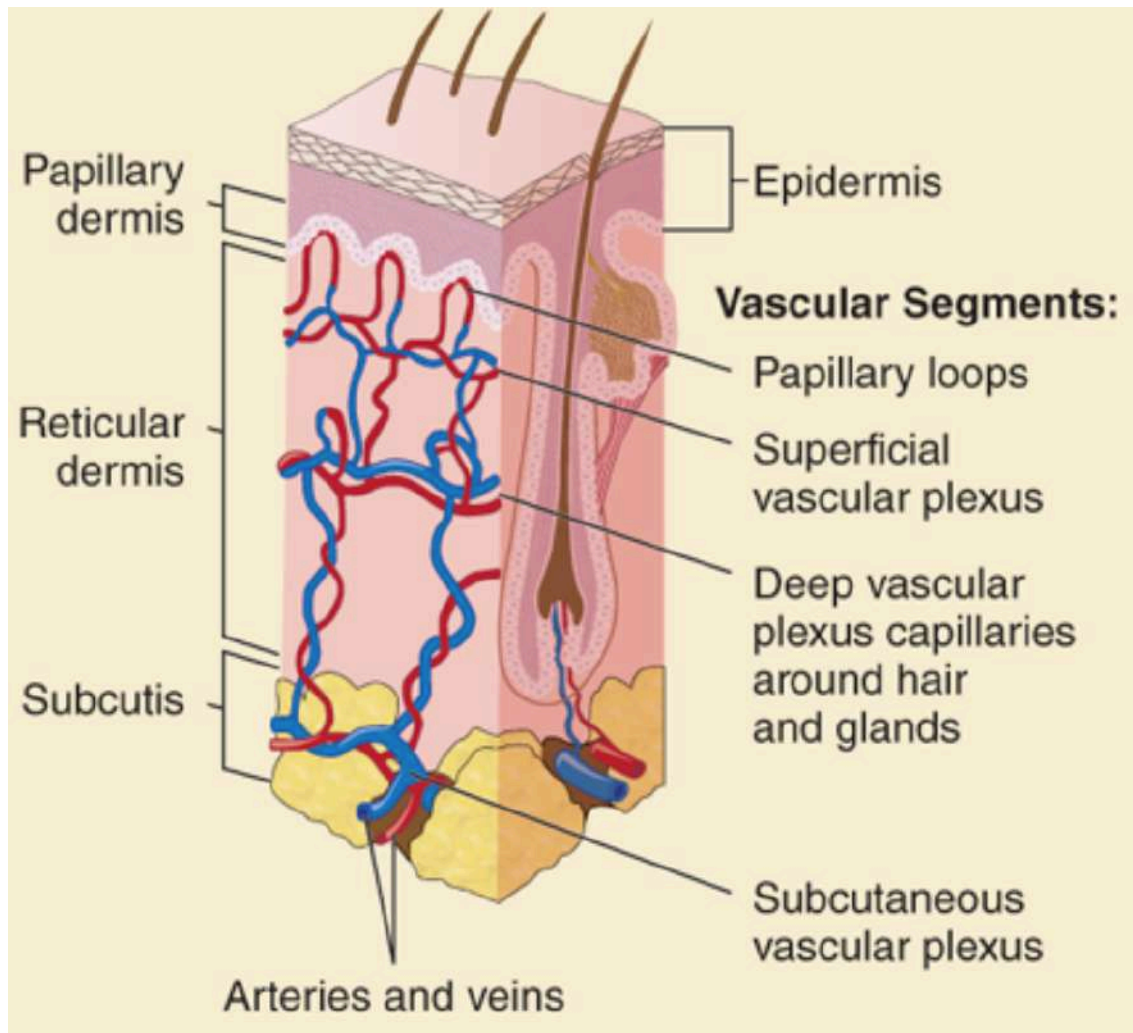


Figure 2. Architecture of skin vasculature (Goldsmith et al., 2012).
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The skin is an important sensorial organ due to the presence of several nervous terminations. Two types of nerve ends in the skin: the free ends that penetrate in basal or spinosum layer and complex nerves that form the Meissner corpuscles, Pacini corpuscles, Ruffini corpuscles and Merkel corpuscles. The Meissner corpuscles are mechanoreceptors concentrated in glabrousness skin surface. Their localization in papillary dermis allows these corpuscles a greater sensitivity to touch and vibration. The Pacini corpuscles are found in deep layers of the skin. These mechanoreceptors detect vibration and pressure. The Ruffini corpuscles are located between the papillary dermis and hypodermis. They are slowly adapting mechanoreceptors that respond to pressure and temperature and register mechanical deformation. The Merkel corpuscles are localized in basal layer of the skin and hair follicles. They detect light touch registering the pression on the skin (Kolarsick et al., 2011; Laverdet et al., 2015).

The two types of glands of the skin are the sweat glands and the sebaceous glands. The first are subdivided in eccrine and apocrine sweat glands (Ballarin and Radaelli, 2012).

The eccrine sweat glands have a tubular structure and are most abundant on the soles of the feet or palms of the hands, and least plentiful on the back. In other mammals, such as carnivores, they found in foot pads or in glabrousness areas. The eccrine glands produce an odorless and clear sweat that has a thermoregulation function (Ballarin and Radaelli, 2012; Baker, 2019; Hodge et al., 2022).

The apocrine sweat glands consist of a glomerulus of secretory tubules and an excretory duct that opens into a hair follicle. They found in deep layer between the dermis and hypodermis. In the human, apocrine glands are main localized to the regions of the axillae, ear canal and perineum. Whereas in mammals, they are spread over the entire body with hair. Their functionality starts during puberty producing a white-yellow and pungent odor sweat that act as pheromones. Mammary, ciliary, and ceruminous glands are specialized modified apocrine glands (Hodge et al., 2022).

The sebaceous glands are made up of an alveolus of secretory tubules and found in the dermis. They are main located on the face and scalp but are absent on the soles of the feet or palms of the hands. Their function is to produce the sebum, an oily and waxy substance, compos of triglycerides, wax esters, squalene, and fat that protects and lubricates the skin and hair (Ballain and Radaelli, 2012; Hodge et al., 2022; Makrantonaki et al., 2011).

The hairs are filaments of keratin produced by hair follicles found in the dermis. The hair follicle consists of an external and internal root sheath and a dermic papilla covered by hair matrix epithelial cells. The hair is med up of three portions: the shaft, the root and the bulbus. The shaft is the visible part that sticks out of the skin. The root extends to deep layers

of the skin and is surrounded by the hair follicle. The bulb is the root end that expand in the hair follicle (Ballain and Radaelli, 2012; Kiani et al., 2017).

The arrector pili muscle extends from dermis to follicle and is responsible for piloerection. Further this muscle, the hair is surround by dense network of capillaries and sensorial innervation (Poblet et al., 2002).

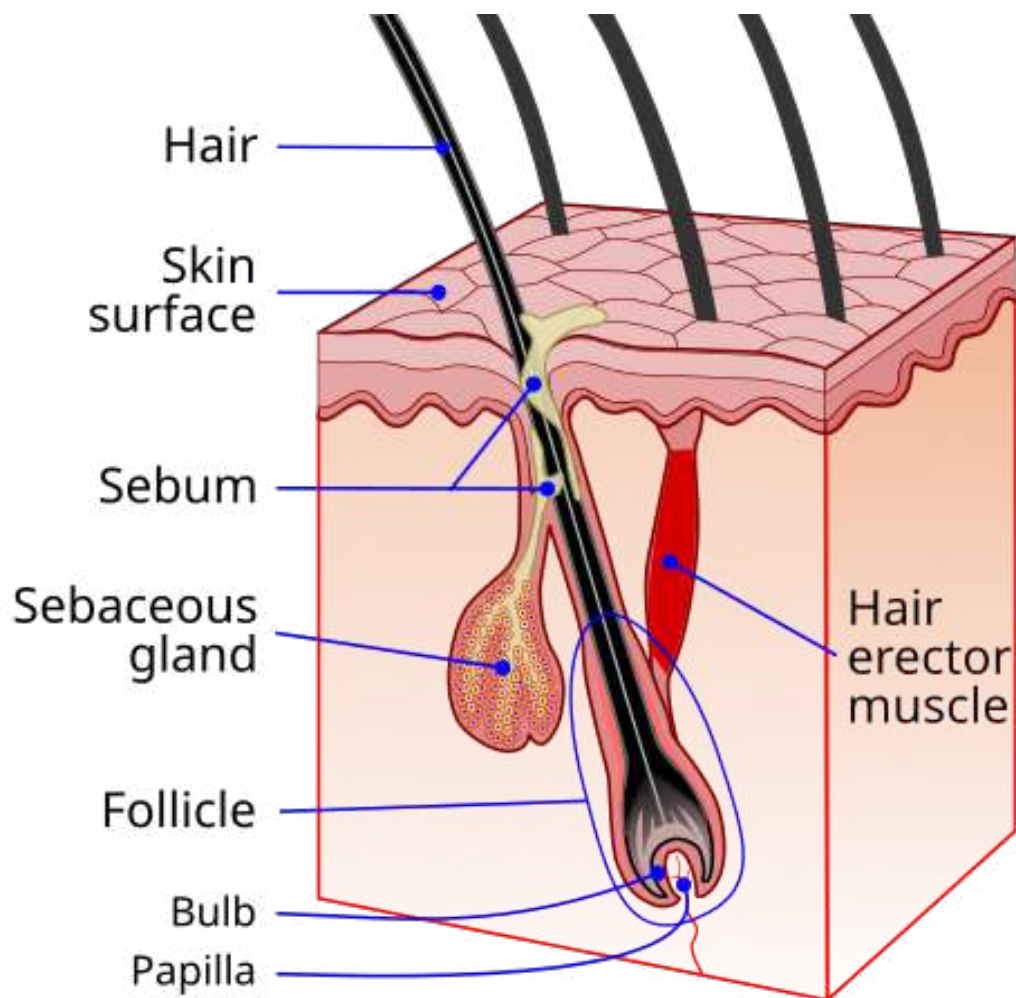


Figure 3. Hair follicle and sebaceous gland (https://en.wikipedia.org/wiki/Sebaceous_gland).

2.2 ANATOMY AND FUNCTION OF THE FORESKIN

The human foreskin, also called as prepuce, is a complex structure of male anatomy. It derived from a middle collision of ectoderm, neuroectoderm and mesenchyme. Its formation starts on the dorsal aspect of glans penis and after glandular urethral formation extends ventrally (Cold and Taylor, 1999).

The foreskin is a bilayer tissue with a surface area of up to 90 cm² and an extension about 51% of the total length of the penile shaft skin (Werker et al., 1998).

It consists of five laminar structures: inner mucosa, lamina propria, dartos muscle, dermis, and outer epithelium (Cold and Taylor, 1999).

The inner mucosa is composed of a simple squamous epithelium, and it is the same that cover the glans penis. It contains Langerhans cells without melanocytes (Cold and Taylor, 1999).

The lamina propria is richly vascularized and does not contain hair follicles, sweat and sebaceous glands. It is characterized by a less compact collagen than glans penis. The ridged band is close the mucocutaneous tip of prepuce and separates the inner mucosa from outer epithelium (Cold and Taylor, 1999).

The dartos muscle is made up of smooth muscle cells covered with elastic fibers. It surrounds the shaft of penis and continues with the scrotal dartos muscle. This muscle allows changes in volume during the erection of penis (Cold and Taylor, 1999).

The dermis of foreskin is formed by connective tissue, blood vessels, nerve bundles, Meissner corpuscles and sebaceous glands. Together with the dartos muscle and frenulum, the dermis anchors the prepuce and promote return it to the anatomically position after erection (Cold and Taylor, 1999).

The outer epithelium is made up of a keratinized stratified squamous epithelium. Melanocytes, Langerhans cells and Merkel cells are found. These last play a crucial role in immune function and tactile stimuli (Cold and Taylor, 1999).

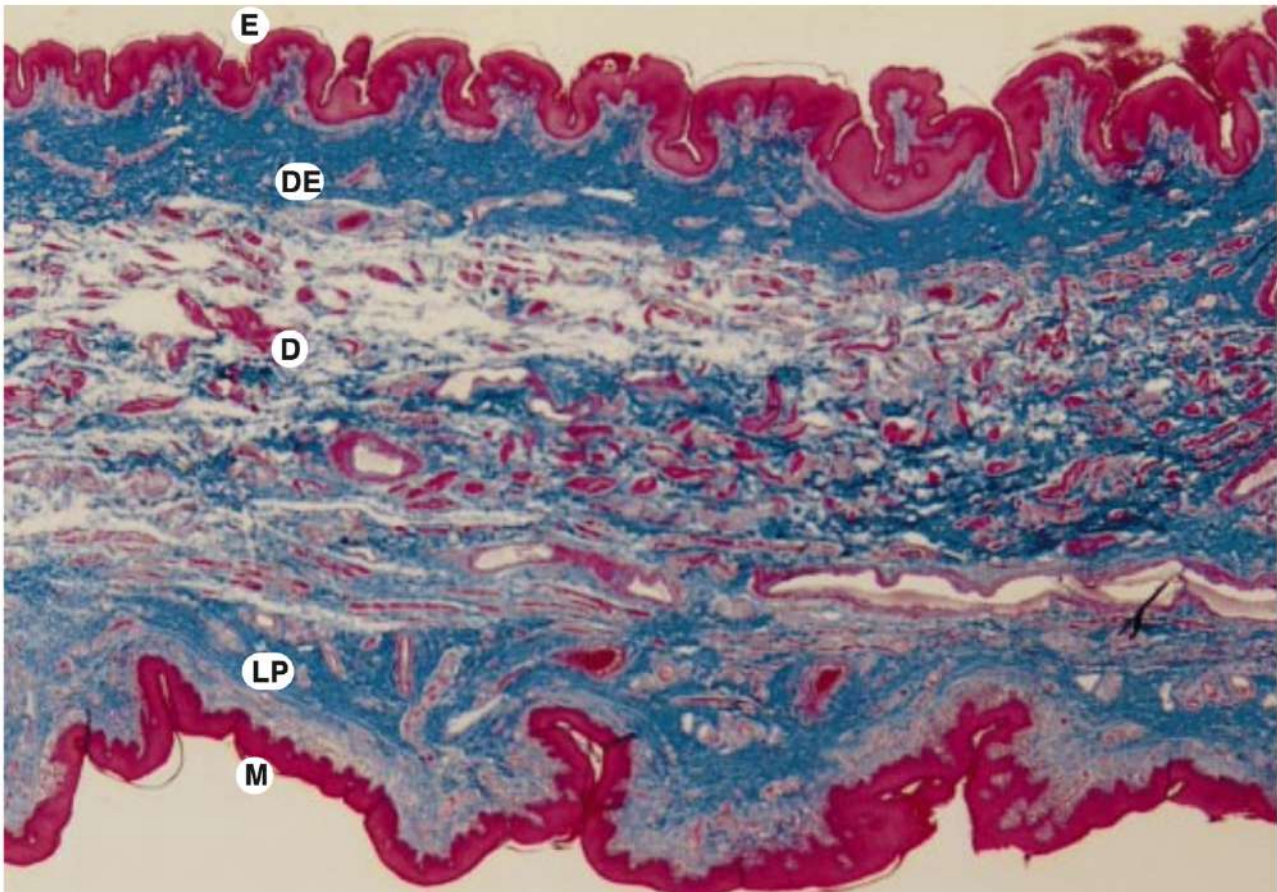


Figure 4. The five layers of male foreskin. Mucosa (M), lamina propria (LP), dartos muscle (D), dermis (DE) and glabrous outer epithelium (E) (Cold and Taylor, 1999).

The foreskin is densely innervated and rich in Meissner corpuscles (most present), Pacini corpuscles and Merkel cells which have a sensory function (Cunha et al., 2020; Nazir et al., 2004).

The autonomic innervation originates from the pelvic plexus, in particular, visceral efferent parasympathetic fibers derive from the sacral plexus (S2-S4) while visceral afferent sympathetic fibers result from the thoracolumbar plexus (T11-L2) (Cold and Taylor, 1999).

The main nerves involved in foreskin innervation are the dorsal nerve of the penis, a branch of the pudendum nerve, and the perineal nerve (Cunha et al., 2020).

The foreskin is supplied by the dorsal artery of the penis, which is a branch of the internal pudenda artery. The blood vessels are axially orientated and located in the superficial fascia that gives off cross-communicating branches to the inner layer. The superficial dorsal vein of the penis drains blood from the prepuce (Perovic and Radojicic, 2003; Özbey et al., 2021).

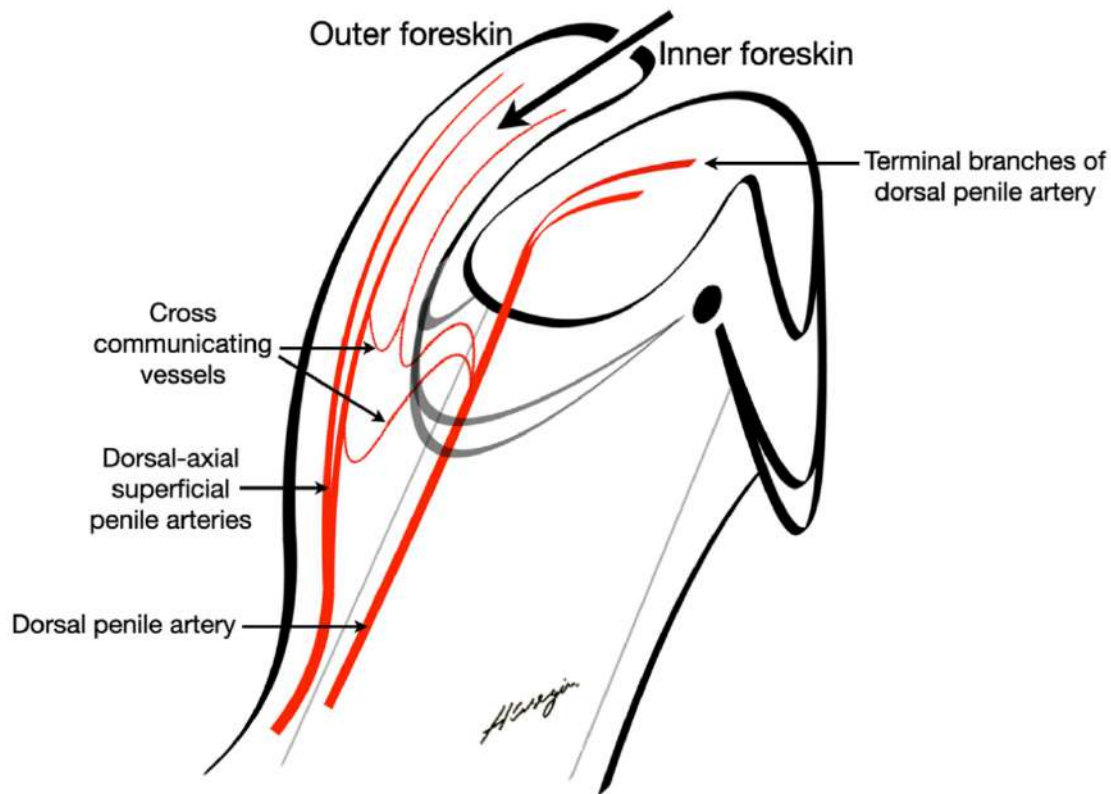


Figure 5. Foreskin arterial blood supply (Özbey et al., 2021).

The foreskin performs many functions. It covers and protects the glans prevent its keratinization. The prepuce acts like a one-way valve stopping the entrance of contaminants and promoting the passage of urine (Dinh et al., 2011).

The cells of Langerhans and other dendritic cells, contained in the foreskin, play a fundamental role in immune system. In addition, the sebaceous glands of prepuce produce lysozyme, chymotrypsin, neutrophil elastase, and cytokine with antibacterial properties. These glands, at level of frenulum, make a natural emollient, known as smegma, that includes prostatic and seminal secretions, desquamated epithelial cells, and the mucin content of the urethral glands. The smegma facilitates erection, preputial eversion, and penetration during sexual intercourse (Fleiss et al., 1998; Almutawa et al., 2022).

Thanks to its dense innervation, the prepuce is an erogenous zone that involved in erection and sexual pleasure. Unlike of glans that contains only free nerve endings, the foreskin is rich in mechanoreceptor (Cunha et al., 2020).

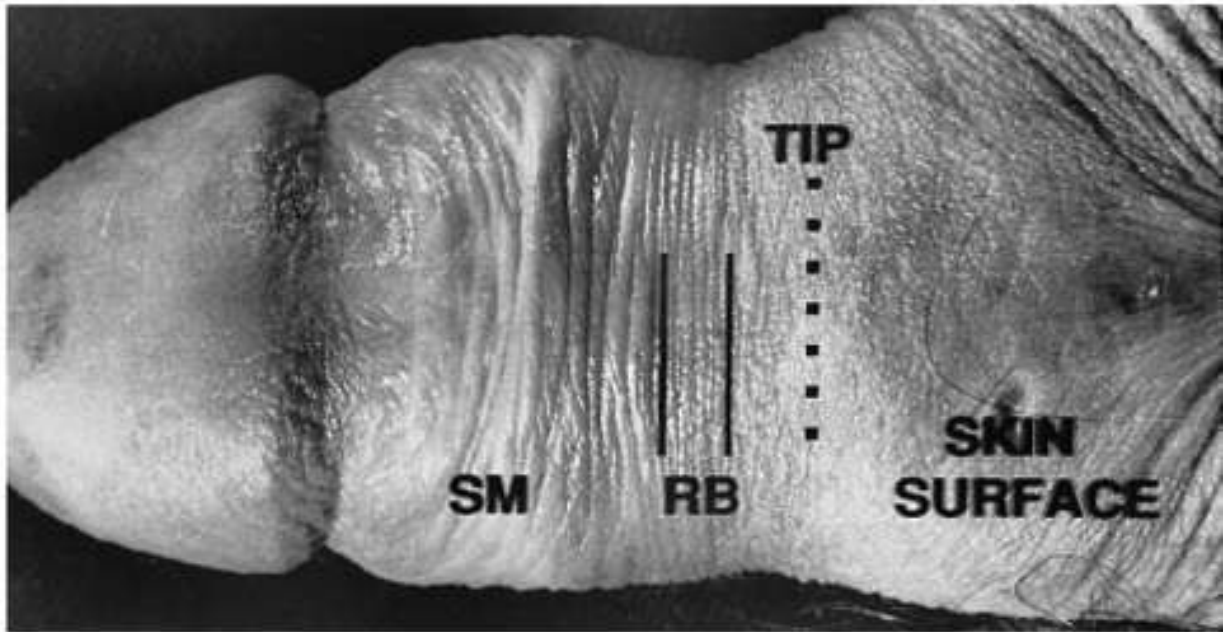


Figure 6. A retracted prepuce in an adult. (SM) smooth mucosa, (RB) ridged band. Dotted line indicates tip of retracted prepuce. External skin surface continuous with skin of shaft of penis (Taylor et al., 1996).

2.3 CIRCUMCISION AND ITS CLINICAL SIGNIFICANCE

The circumcision is the surgical removal of the foreskin that covering the glans of the penis (Raynor, 2010).

It is one of the most surgical techniques performed around the world, just considered that globally about 38% of people and 2.6% in Italy are circumcised. The circumcision is widespread among the Jewish and Muslim populations, and there is a high rate of circumcised in the United States. In contrast, routine circumcision is rarely performed in Europe, China, the Far East, and Central and South America (Ventura et al., 2020; Raynor, 2010).

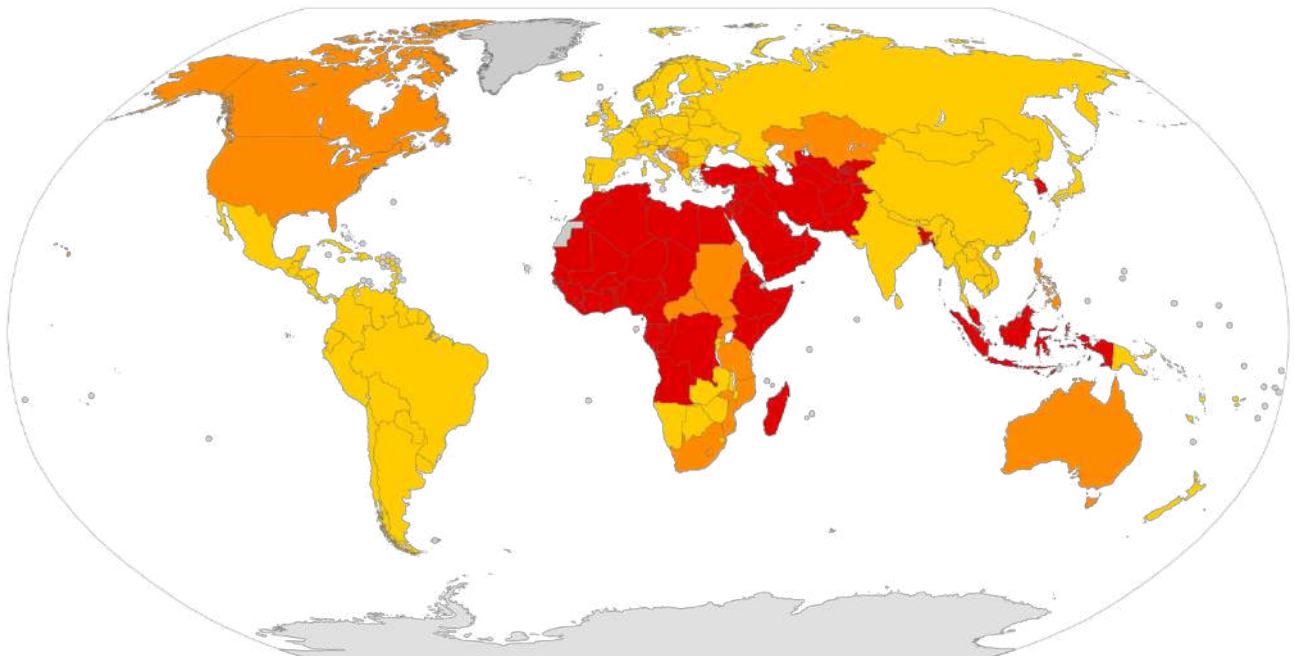


Figure 7. Global map of male circumcision prevalence. Red: widespread, near universal: >80% prevalence. Orange: widespread, common: 20–80% prevalence. Yellow: Uncommon: <20% prevalence. Grey: N/A (https://en.wikipedia.org/wiki/Prevalence_of_circumcision#).

The removal of the prepuce was already reported in ancient times evidence dating the practice to Egyptians. To date, the circumcision is performed for religious, cultural, medical, personal preference and several other reasons (Raynor, 2010).

The circumcision generates much controversy and different researchers seek to understand in what ways, medically speaking, this procedure can bring benefits or cause harm (Friedman, et al., 2016).

It is reported that the circumcision protects against the risk of human immunodeficiency virus (HIV) because the inner surface of the foreskin is rich in Langerhans cells with HIV receptors. These observational studies were performed in developed countries which present significant biases. Other results showed no significant correlation between circumcision and HIV, especially in developed countries. For example, the United States has the highest rate of HIV and also the highest rate of infant circumcision between developed countries (Tian et al., 2013; MacNelly, 2007).

Studies have showed that circumcision reduce the incidence of sexually transmissible disease like, syphilis, gonorrhoea, candidiasis, genital herpes and human papilloma virus. This last can be responsible of penile cancer. However, other studies have found little support for or have refuted these findings altogether (Friedamn et al., 2016).

The circumcision is available to reduce the incidence of urinary tract infections (UTI) in infants even if it is low in the first year of life. It is estimated that the rate of UTI must equal or exceed 29% for neonatal circumcision to be cost effective (MacNelly, 2007).

The foreskin is a complex structure rich in nerve endings which plays an important role in sexual pleasure and activity. Some papers reported that the circumcision adversely affects sexual function in many men causing a decrease in masturbatory pleasure and sexual enjoyment. Other papers described no differences in sexual desire, dyspareunia, premature ejaculation, ejaculation latency time, erectile dysfunctions and orgasm difficulties between circumcised and uncircumcised males suggesting that the circumcision does not have adversely affect sexual functions (Kim and Pang, 2007; Taylor et al., 1998).

In addition to physical alterations, the infant circumcision may have psychological adverse outcomes. It has been sometimes defined as a traumatic and painful event. Early-circumcised men showed higher attachment insecurity and emotional instability, lower empathy and trust, higher sexual libido with unrestricted sociosexuality, and higher stress and risk-taking attitudes (Goldman, 1999; Aydoğdu et al., 2022).

The Circumcision Research Center reported that the feeling described by circumcised men include anger, sense of loss, shame, sense of having been victimized and violated, fear, distrust, grief, and jealousy of intact men (Goldman, 1999).

The pain relates to infant circumcision may lead adverse changes to brain structure and function in the prefrontal cortex that impact adversely on a child's subsequent personality development. It was reported that the circumcised infants are predisposed towards higher negative mood states such as anxiety, stress, and depression (Boyle, 2015).

Some diseases make the circumcision necessary such as the inability to retract the foreskin due to narrowing of its opening known as phimosis, balanitis xerotica obliterans that is a

progressive sclerosing inflammatory dermatosis of the glans and prepuce or paraphimosis that is characterized by the inability to pull the retracted foreskin back over the glans (Hayashi et al., 2015).

The non-therapeutic infant circumcision remains contentious and hotly debated, and well designed and prospective studies are required for more clarity however it is important not to underestimate the psychological sequelae correlated to this procedure (Friedman, et al., 2016; Hayashi et al., 2015).

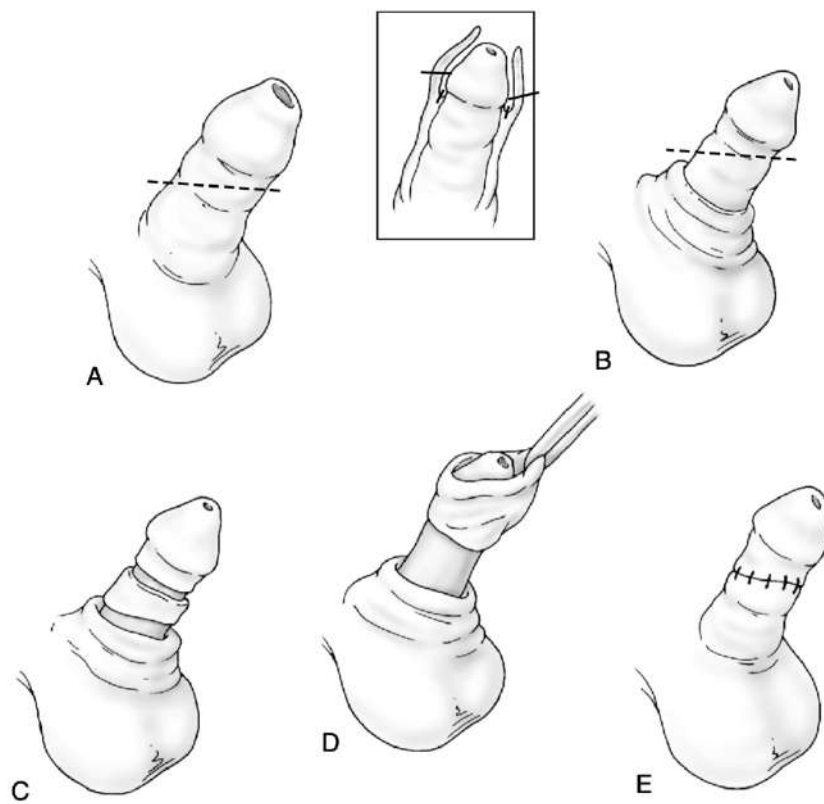


Figure 8. Schematic representation of freehand circumcision: (A) The initial incision is made in the shaft skin, leaving more skin ventrally. (B) A second incision is then made in the subcoronal sulcus, leaving a generous cuff. The inset shows the amount of foreskin to be excised. (C-D) The isolated foreskin is then excised. (E) The shaft skin is sutured to the subcoronal skin (Raynor, 2010).

2.4 FORESKIN RESTORATION

The first efforts to restore foreskin took place in Ancient Greek society as a reflection of their specific standards for the human physique (Hodges, 2001).

Since then, there have been proposed different techniques however the available data on treatment options remains sparse. They are essentially divided in two methods: surgical and non-surgical (Timmermans et al., 2022; Kennedy, 2015; Collier, 2011)

The first one reported method included different conservative procedures using traction. A physician described the application of emollient prior to the traction (Hodges, 2001).

In alternative, another old technique is the kynodesme which is a cord tied tightly around the most distal and tubular portion of the foreskin (Timmermans et al., 2022; Hodges, 2001).

Several surgical techniques are reported for the reconstruction of foreskin. These treatments provide the use of cutaneous and fascial layers (Gupta et al., 2021; Timmermans et al., 2022; Schultheiss et al., 1998). The ancient Romans described a surgical technique for circumcised men that involved in a subcoronary incision, and a full thickness dissection was performed of the penile shaft. The elastic penile skin would be pulled over the glans, ligated at the distal neoprepuce and a non-adherent lead-oxide plaster would be applied to the single layered skin to prevent adhesion to the glans and promote epithelization (Timmermans et al., 2022; Rubin, 1980).

Afterwards, Tushnet described three methods to restore the foreskin. The first was to pull over the skin from behind the corona, scarifying and suturing the scarified edges in order to simulate a phimosis. This procedure had a high incidence of infectious and retraction of the new foreskin. The second method was a variation of the technique proposed by ancient Romans. The third method involved in a circular incision around the preputial area and the separation of wound. In the second place a long oblong skin graft was excised from the iliac crest. The narrow edges of the strip were sutured together and folded longitudinally to create an inner and outer lining. This surgery was reported only in three men (Tushnet, 1965; Hodges, 2001).

Greer proposed a four-stage procedure. First, a circular incision was made at the base of the penis, dissected, and turned over to create a cylindrical single layer flap that would become the inner lining of the neoprepuce. The bipedicle scrotal flap was then elevated and the shaft was placed in the scrotal tunnel. After healing, which could take months, the penis was released and closed accordingly (Greer et al., 1982).

Surgical reconstructions using fascial layers was proposed by Brandes. He described two methods using the fascial or a fasciocutaneous circular penile flap. The flaps were collected

from the distal penis and rely on the highly vascularized deep fascia of penis (Brandes and McAninch, 1999).

Recently, Gupta et al., reported the novel method which consisted of penile degloving and maintenance of neo prepuce, with the help of de-epithelization of glans penis and a few key sutures (Gupta et al., 2021).

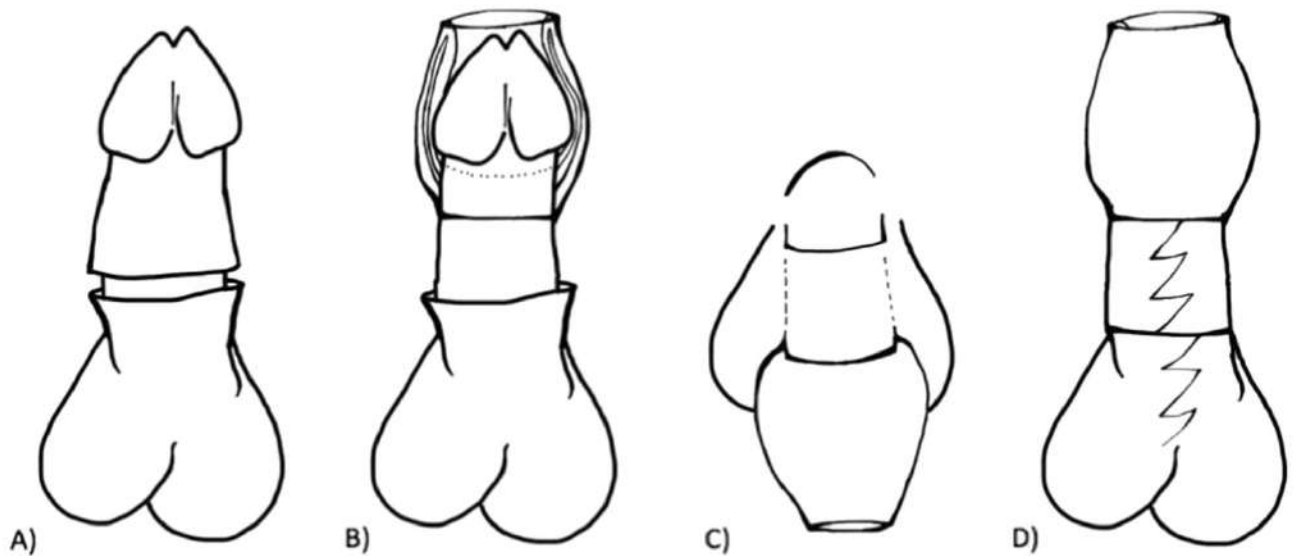


Figure 9: Surgical technique for foreskin restoration described by Goodwin: (A) Circular incision of the skin. (B) Mobilizing the skin distally to create a double-layered neoprepuce. (C) Burying the denuded shaft in the scrotal tunnel. (D) Release of the penile corpus using Z-plasty. (Goodwin, 1990).

Non-surgical methods can be divided in taping and tapeless methods. They are based on manual stretching and device-assisted tissue expansion.

The first modern taping method was introduced by the Brothers United for Future Foreskins in the early eighties. It is made up of cross-taping the prepuce over the glans and ring-taping it at the base of the intended neo prepuce (Timmermans et al., 2022). Another taping method is MTC-taping. It consists of T-shaped tape that is applied around the base of the glans, pulled distally, and clipped or tied to an elastic band. In addition, weights could be used if the skin is sufficient (Timmermans et al., 2022).

The tapeless method involved in gripping the foreskin and penile skin and stretching it distally. Among these methods are Tapeless Conical (TLC) tugging that is a two cone-shaped

component, which entraps the residual prepuce. Traction is achieved using an elastic band, weights or strapping it to the upper leg.

Other methods provide the use both pushing the glans whilst and pulling the skin simultaneously such as the Dual-action Incremental Longitudinal Expander insert (DILE-insert) (Timmermans et al., 2022).



Figure 10. TLC tugger and additional strapping to the upper leg for non-surgical foreskin restoration (TLCTugger.com; Timmermans et al, 2022).

The non-surgical methods have yielded good results with minimal adverse effects, but they are though time consuming. However, these techniques of restoration are poorly reported in literature and the effective outcomes have not been scientifically described. These methods are prevalently mentioned on web-based forums (Timmermans et al., 2022; Collier, 2011).

In contrast, the surgical methods frequently produce unsatisfactory results, because the neo prepuce differs from original foreskin in the color and functionality (Timmermans et al., 2022; Collier, 2011; Purpura et al., 2018).

The foreskin is a very complex and specialized structure richly vascularized and innervated that is difficult to reconstruct with autografts (Purpura et al., 2018).

To date, there is not a treatment protocol for foreskin restoration. Non-surgical methods are the therapeutic choice that gives higher cosmetic appearance and functionality than surgical

methods without complications (Timmermans et al., 2022; Collier, 2011; Purpura et al., 2018).

2.5 DECELLULARIZED EXTRACELLULAR MATRIX: A PROMISING STRATEGY FOR FORESKIN REGENERATION

In this scenario, the regenerative medicine and tissue engineering could be using to develop new methods to obtain definitive foreskin reconstruction. In particular, decellularized extracellular matrix (dECM) biomaterials have become increasingly popular for the repair and regeneration of the skin, tissue and organs in the last years (Golebiowska et al., 2024).

The aim of dECM biomaterials is to capitalize on the properties of native ECM and promote organized regeneration of host tissue (Mendibil et al., 2020).

ECM is a large network consisting of extracellular macromolecules and minerals, such as collagen, hyaluronic acid, proteoglycan, glycosaminoglycan, enzymes, glycoproteins, fibronectin, elastin, laminin, various cell growth factors and hydroxyapatite that surround, support, and give structure to cells and tissues in the body. It is directly involved in tissue repair provides physical support for cell adhesion and significantly influences cell behaviors including migration, proliferation, and differentiation (Yao et al., 2019).

Some kinds of bioactive peptides present in ECM, such as collagen and fibronectin, have a diverse array of biologic activities including angiogenesis, antimicrobial effects, and chemotactic effects. In addition, ECM acts as a niche for stem cell differentiation (Brown and Badylak, 2014).

These scaffolds based on extracellular matrix are able to proliferate and promote the growth and differentiation of many types of cells as well as inducing structural tissue remodeling processes after transplantation (Gierek et al., 2022).

Decellularized extracellular matrix biomaterials are very promising for skin repair and regeneration, and they are known as acellular dermal matrix (ADM). The first use of ADM in regenerative medicine is in the treatment of burn injuries and wound management. Other common application fields are in the reconstructive breast surgeries, hernia repair and gynecological reconstructive surgery (Gierek et al., 2022; Petrie et al., 2022).

Skin

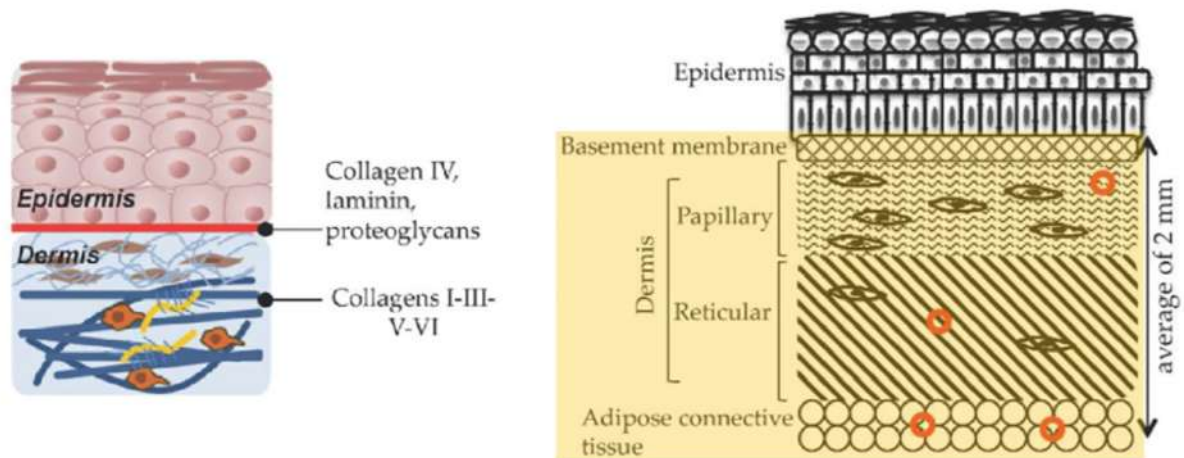


Figure 11. Area harvested for processing acellular dermal matrix (yellow highlighted) (Petrie et al., 2022).

dECM scaffolds used for skin repair can be classified based on the origin of ECM in organ/tissue and cell-derived dECM (Jiang et al., 2023).

dECMs derived from organ/tissue preserve the natural three-dimensional structure promoting the regulating cell adhesion, proliferation, differentiation, and other behaviors and ensuring good mechanical strength, however, they have disadvantages including immunogenicity, cell permeability and potential disease transmission (Jiang et al., 2023).

dECMs derived from cells consist of a complex and organized mixture of macromolecules that can mimic native tissue microenvironments (Fitzpatrick and McDevitt, 2015).

They can be customized by selecting the type of cells used to generate ECM, the culture system, the application of external stimuli to modulate ECM production and have the ability to genetically modify the source cells to augment or silence the expression of target molecules (Fitzpatrick and McDevitt, 2015). Cell-derived ECMs have fewer immunogenic components and less potential for pathogen transmission. In contrast, they are deficient in a three-dimensional structure, which requires the addition of additional molecules or combination with other scaffold materials to increase mechanical strength in clinical applications (Jiang et al., 2023).

dECMs derived from organ/tissue can be classified in turn into animal or human derived dECM. The first have major immunogenic and biological risks for this reason their application is limited (Jiang et al., 2023).

In decellularized ECM scaffolds, the antigenic component such as cell membrane, nucleic acids, and mitochondria are removed to avoid or reduce host immune rejection and inflammatory response (Neishabouri et al., 2022).

It was described the minimum criteria assessing the safety of decellularized tissue: (1) less than 50 ng double-stranded DNA (dsDNA) per mg ECM dry weight, (2) less than 200 bp DNA fragment length, and (3) no visible nuclear material by 4',6-diamidino-2-phenylindole (DAPI) staining (Crapo et. al., 2011).

Several methods of tissue and organ decellularization have been reported. These methods can be roughly divided into three main categories: biological decellularization methods, chemical decellularization methods, and physical decellularization methods. (Heath, 2018)

The biologic methods involve in enzymatic treatment of tissue. It offers high specificity for the removal of cell components and undesirable ECM constituents. The most enzymes use for the decellularization of tissue and organ are the trypsin and nuclease (Zhang et al., 2022).

The physical methods use stress to result cell lysis without significantly disrupting the ultrastructure of the tissue (Kim et al., 2018). Especially, the freeze-thaw is one of the most used physically treatment (Zhang et al., 2022). It consists of a repetitive freeze-drying in nitrogen and subsequent thawing in butter solution (Zhang et al., 2022). Perfusion, immersion and agitation represent other methods to obtain the physical decellularization of tissue.

The physical methods preserve more than other methods the ECM architecture, however, they alone cannot completely remove cellular debris from the tissue (Kim et al., 2018).

The chemical methods treat the tissue with acid or basic conditions or detergents. Acids or bases treatment results in degradation of cells and the removal of cellular components such as nucleic acids. The degree of decellularization is related to the type and concentration of the acid or base being used, processing time, and the type of tissue being treated. The bases are more aggressive for the tissue and cause a significant loss of mechanical properties. For this reason, their use is limited in particularly cases where reduction of structural components is a desired result (Kim et al., 2018). In addition, chemical decellularization can be obtain using detergents that disrupt the hydrophobic-hydrophilic interactions among molecules (Zhang et al., 2022). Three main types of detergents are used: nonionic, ionic, and zwitterionic.

Another popular chemical method is osmotic lysis that allows the ruptures of plasma membrane via osmotic shock after the immersion in a hypertonic or hypotonic solution.

In the last years, new treatments such as vacuum-assisted and apoptosis-assisted decellularization are described. Due to complex mechanism, these novel methods have not been applied extensively (Zhang et al., 2022).

The combination of methods may improve decellularization efficiency and limit the negative effects of protocols based on single agents (Dussoyer et al., 2020).

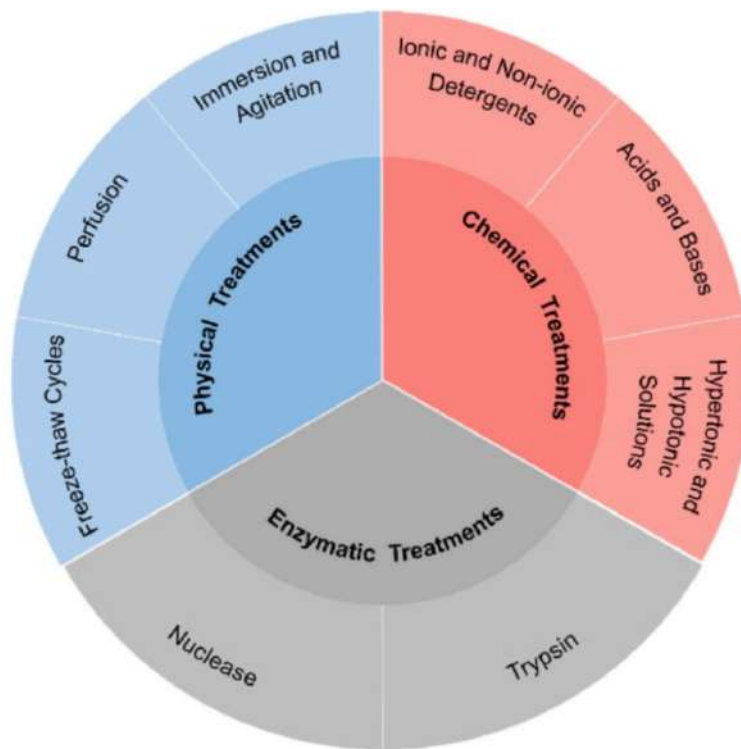


Figure 12. Three different decellularization methods: physical, chemical, and biologic(enzymatic) (Zhang et al., 2022).

The aim of decellularization methods is to remove cells from tissues and retain the integrity of the ECM and microstructure (Luo et al., 2023).

Complete removal of immunogenetic components is often difficult, and the cell residues of dECM may cause an inflammatory response and eventually lead to foreign body reaction (Jiang et al., 2023).

In the 2018, Purpura et al have developed a decellularized extracellular matrix–based biomaterial scaffold derived from human foreskin.

The human foreskin was harvested from living donors for therapeutic purpose. After the harvesting, the foreskins were immersed in a freezing solution composed of RPMI 1640 medium with the addition of antibiotics and 10% cryoprotectant and subsequently frozen for storage at -80°C .

The decellularization of foreskin was performed in various phases. Firstly, the dermal and epidermal layers of the tissue were physically separated using 2.5% trypsin, diluted to 1× with 0.9% NaCl saline solution.

The isolated dermal tissue was placed inside cell culture flasks and covered with 2.5% trypsin, diluted to 4× with 0.9% for 24 hours. During this process, the foreskin was situated in an incubator with a controlled atmosphere and temperature (5% CO_2 /air and 37°C).

Successively, the samples were washed in sterile 0.9% NaCl saline and dipped in RPMI medium (containing 10,000IU/mL penicillin, 10mg/mL streptomycin, $25\mu\text{g/mL}$ amphotericin B) for 15 min and sealed in sterile cryofreezing, without the addition of cryoprotectant.

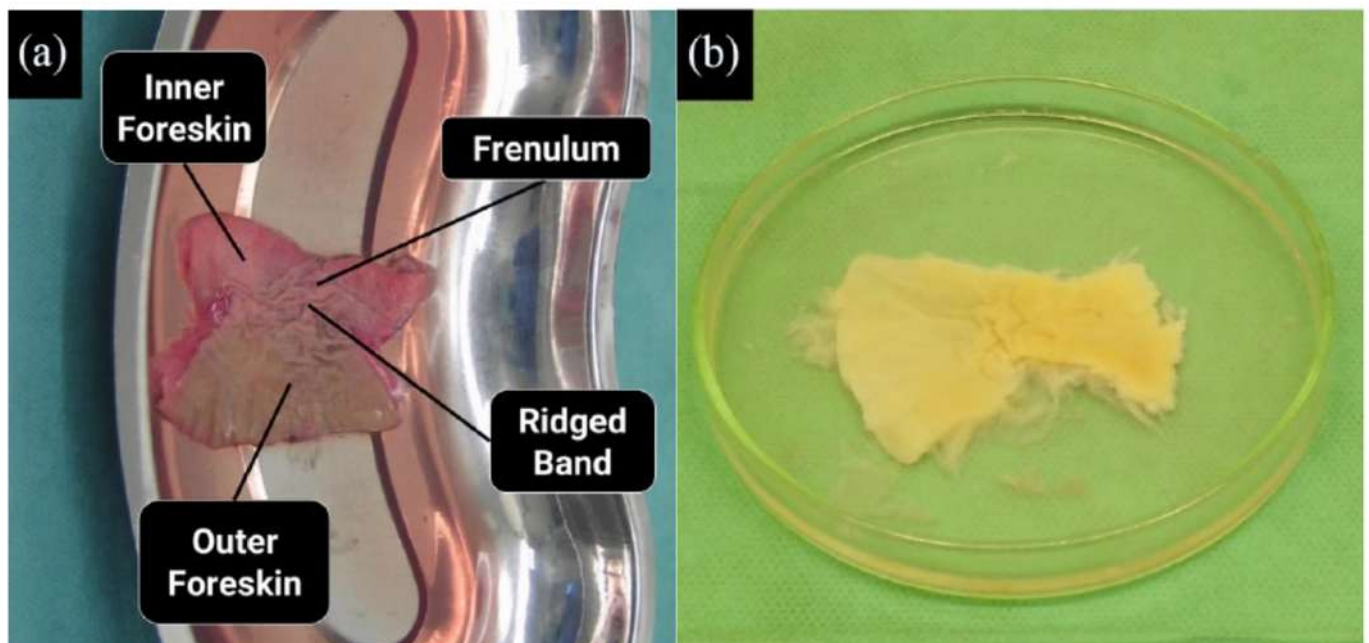


Figure 13. Appearance of human foreskin tissue: (A) Fresh frozen foreskin tissue. (B) Decellularized foreskin tissue (Purpura et al., 2018).

After the decellularization process, the resulting matrices underwent to a variety of assay. The decellularized foreskin showed maintenance in the architecture and structural integrity of the dermal layer with a compact and well-preserved ECM.

The cell viability was drastically reduced when compared against the fresh frozen foreskin.

In addition, the decellularized foreskin had a high level of fibroblastic growth factor (FGFb) that is involved in the regeneration of a wide variety of tissue types such as skin, blood vessel, muscle, adipose, tendon, ligament, cartilage, bone, tooth, and nerve tissues.

This decellularized scaffold is very interesting for a novel approach to foreskin reconstruction and regeneration due to the presence of some intrinsic anatomic and structural components that are biologically inherent in the foreskin tissue (Purpura et al., 2018).

A more recent study compared the efficacy and feasibility of two different protocols for foreskin decellularization. Both enzymatic and detergent methods conserved the ultrastructure and composition of natural ECM while being DNA-free and non-toxic (Novotna et al., 2023).

The results of these research, however, need to be supported by in vivo studies.

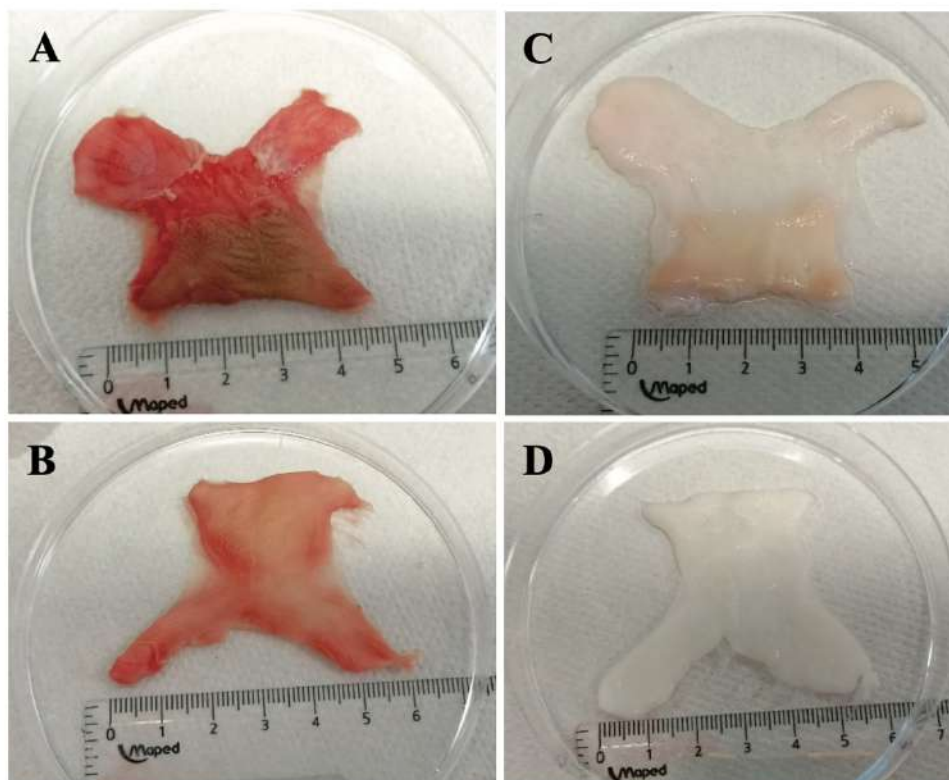


Figure 14. Appearance of human foreskin tissue: (A-B) Fresh frozen foreskin tissue. (C) Foreskin tissue after enzymatic treatment. (D) Foreskin tissue after chemical treatment (Novotna et al., 2023).

3. EXPERIMENTAL STUDY

Foregen Onlus is a no profit association founded in 2010. The mission of Foregen Onlus is to heal the physical and psychological damage inherent to circumcision through tissue engineering techniques, which offer outstanding potential to regrow human tissue, especially dermal tissue, lost in this procedure.

Foregen Onlus in cooperation with Emilia Romagna Regional Skin Bank have patented a new technique of decellularization to obtain an extracellular matrix scaffold for the reconstruction of human foreskin (Purpura et al., 2018).

In vitro results are very promising as definitive treatment for complete foreskin regeneration.

The next steps will involve the assessment of scaffold proprieties in animal model and subsequently in human patient.

The study described in this thesis aims to evaluate the immune response and the integration of decellularized foreskin matrix produced by the Emilia Romagna Regional Skin Bank when implanted in the host (rat). In particular, the type and the degree of inflammatory infiltrate, the neovascularization and the eventually foreign body reaction will be assessed.

This study, conducted at University of Camerino, was approved by Ministry of Health with protocol number 424/2021-PR.

4. MATERIAL AND METHODS

4.1 ANIMALS

The study involved twenty-six Wistar male rats weighing approximately 350 gr and 4 months of age.

The subjects were housed in separate cages (two rats for each cage) with artificial day-night cycle (12 hours light/dark), constant temperature (20-22°C) and humidity (45-55%).

The rats were randomly divided in two groups of the study (A group = 13 rats; B group = 13 rats). The subjects of A group were involved in the study for 30 days while those of B group for 5 days.

4.2 SURGICAL PROCEDURE

After subcutaneous administration of buprenorphine (50 mcg/kg) and carprofen (5 mg/kg), all rats underwent to general anesthesia by intraperitoneal administration of ketamine (70 mcg/kg) and xylazine (10 mg/kg). Furthermore, additional oxygenation was ensured with the administration of pure oxygen via a face mask.

When a good anesthesiologic plan was achieved, the subjects were positioned in sternal recumbency and a square infrascapular area of about 6 cm² was clipped (Figure 15).

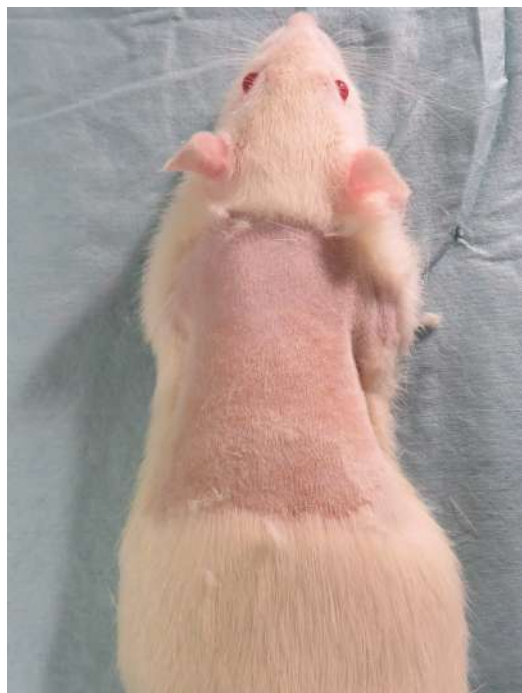


Figure 15. Trichotomy of infrascapular region.

Preoperative skin preparation was performed with 10% povidone-iodine and alcohol (Figure 16).



Figure 16. Preoperative skin preparation.

Under complete aseptic precautions, an infrascapular skin incision of about 1 cm was made and a decellularized extracellular matrix scaffold (about 2 cm of diameter) derived from human foreskin was implanted in hypodermal layer (Figure 17; Figure 18). The scaffolds were produced by Emilia Romagna Regional Skin Bank as Purpura et al. previously described (Purpura et al., 2018).



Figure 17. Aseptic preparation of surgical field.



Figure 18. Decellularized foreskin implantation in hypodermic layer of a rat.

After the implantation of scaffold, the skin was sutured using a USP 4/0 absorbable monofilament thread (Figure 19).



Figure 19. Suture of skin incision.

The rats were heated infrared lamp during the awakening phase. Heating support was discontinued when the rectal temperature was above 37.5 °C (Figure 20).



Figure 20. Measurement of rectal temperature.

Other physiological parameters monitored were heart and respiratory rate and the degree of sedation until the rats were fully awake (Figure 21).

The Rat Grimace Scale was used to evaluate the pain degree and the discomfort in postoperative period.

The rats received subcutaneous administration of buprenorphine (50 mcg/kg) and carprofen (5 mg/kg) every 24 hours for 2 days and enrofloxacin (0.2mg/ml) dissolved in drinking water for 5 days. During the first five days postoperative, the subjects underwent to daily clinical examination to assess the health state and physiological functions.



Figure 21. Assessment of heart rate.

4.3 CLINICAL ASSESSMENTS

The infrascapular region (implant site) was clinically monitored to detect the key signs of inflammation.

The clinical evaluations of all subjects (both B and A groups) were performed daily (T0, T1, T2, T3, T4, T5) for the first 5 days post-op, subsequently each 5 days (T10, T15, T20, T25, T30) until 30 days post-op just in A group.

Specifically, heat (*calor*), redness (*rubor*) and swelling (*tumor*) were assessed by 4 points score from 0 (no signs) to 3 (severe signs of inflammation).

- *Calor*: 0 indicated that the temperature of infrascapular region was the same of the body skin, 1 a slight increase of the temperature, 2 a moderate increase of the temperature and 3 a very increased of the temperature. The score was established to palpate the mentioned regions.
- *Rubor*: the absence of infrascapular skin redness was classified as 0, a slight reddening of the skin at the surgical site as 1, reddening of the skin not exceeded two millimeters as 2 and reddening of the skin at the surgical site that exceeded two millimeters as 3.
- *Tumor*: an increasing of the infrascapular skin thickness less than 2 millimeters compared to the preoperative thickness was rated as 0. An increasing between 2 and 4 millimeters as 1, an increasing between 4 and 6 millimeters as 2 and an increasing more than 6 millimeters as 3. The skin measurement was performed by a manual caliber (Figure 22).



Figure 22. Manual caliber to skin measurement thickness.

4.4 HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS

The subjects were euthanized at 5 days (B group) and 30 days (A group) post implantation of scaffold. All rats underwent to general anesthesia by intraperitoneal administration of ketamine (70 mcg/kg) and xylazine (10 mg/ kg) and the euthanasia was performed through carbon dioxide (CO₂) inhalation.

A skin infrascapular biopsy about 6 cm of diameter was obtained. The skin scar was considered the center of biopsy (Figure 23; Figure 24).

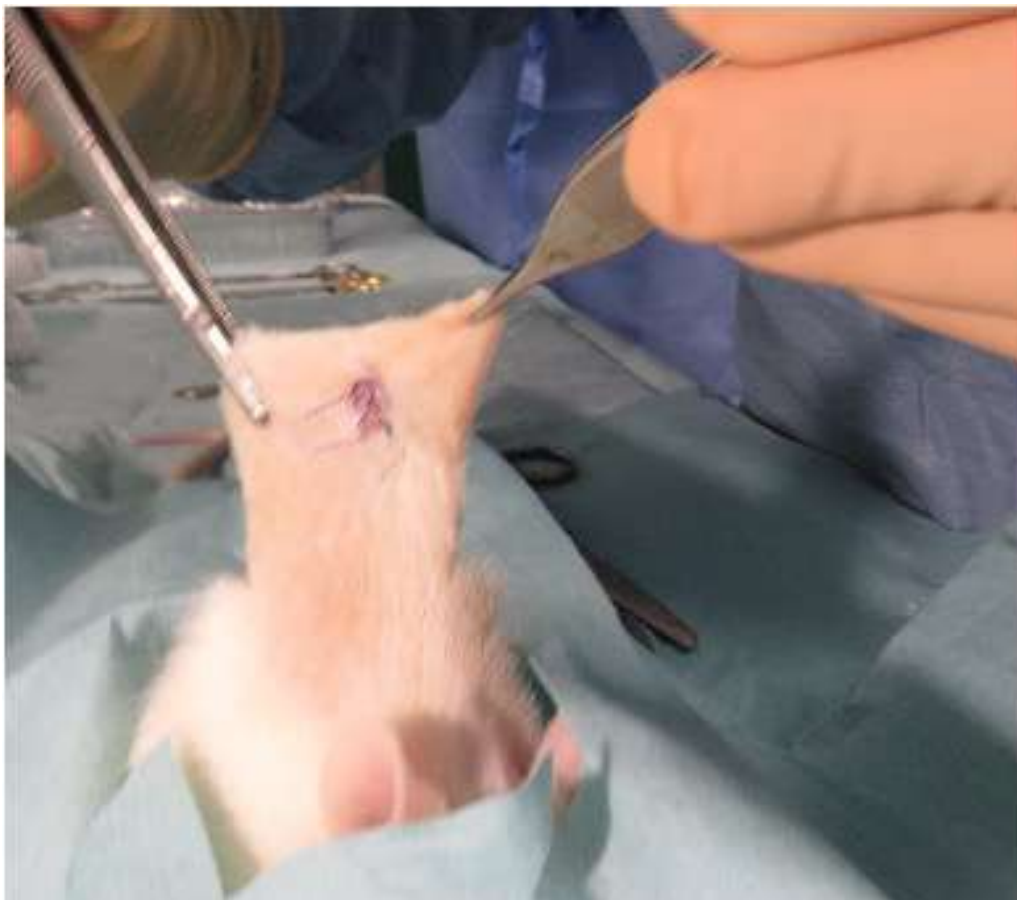


Figure 23. Execution of a skin infrascapular biopsy.



Figure 24. Explanted scaffold with some neo microvessel (white arrow).

The samples were stored in 10% buffered formalin and positioned in histological processing cages, then immersed in paraffin and cut with Leica microtome into thin 3 μm histological sections. The sections were then set onto electrostatic slides (Histoline, Milan, Italy) for maximum adhesion, allowed to dry and subsequently stained with Hematoxylin-Eosin. Histological and immunohistochemical assessment was performed blindly by a single operating pathologist.

Histological examination of decellularized foreskin matrix included assessment of neutrophils, eosinophiles, lymphocytes, macrophages, and cell colonization (fibroblasts) according to the following score: from 0 to 3 inflammatory cells per field at 40x (score 0); from 3 to 5 cells per field (score 1); from 5 to 15 cells per field (score 2); over 15 cells per field (score 3).

The samples were deparaffinized with successive xylene baths and then rinsed them three times in pure alcohol and washed them in solutions with decreasing alcohol concentration (95% and 85%). before proceeding to incubation of the primary antibodies for Factor VIII

and Vimentin. The endogenous peroxidase activity was blocked by using hydrogen peroxide (3%) in methanol for 20 minutes then washed them 10 times with distilled water. Following the peroxidase blocking, nonspecific binding was blocked with 3% milk powder for 1 hour in a 27°C incubator, then rinsed the slides with TRIS solution. After dewaxing, the sections were set in EDTA buffer, pH 9.0, and then in a microwave oven at 750 W for two cycles of 11 minutes to promote antigenicity. The slides were left to cool at room temperature for at least 20 minutes prior to incubation with goat normal serum (Sigma), further processing for primary antibodies incubation.

The primary antibodies were the mono (mAbs) and polyclonal (pAbs). Specifically, mouse mAb anti-vimentin (Dako, Glostrup, Denmark, clone V9), and rabbit pAb anti-Factor VIII related antigen (CliniSciences, Guidonia, Italy, Cat# RP012-05) were used.

Immunohistochemical evaluations included, FVIII positive-microvessel neo-formation, and the presence of vimentin positive stained fibroblasts forming capsule surrounding the scaffold. The scoring of semiquantitative assessment regarding the immunohistochemical reaction in the antigens was as following: 0 (absence of antigen expression), 1 (weak and spotted antigen expression), 2 (weak but profuse antigen expression across the whole specimen), and 3 (profuse and marked antigen expression).

5. STATISTICAL ANALYSIS

A sample size calculation was performed considering previous data in rats (Prudente et al., 2016). Power calculation was conducted for a two-tailed t-test with a power of 0.95 and an alpha error of 0.05 (G*Power Version 3.1.9.3). This suggested that a minimum of 13 rats per group could be sufficient to detect significant differences (G*Power v. 3.0.10; University of Düsseldorf, Germany) (Faul et al., 2009). Statistical analysis was performed using MedCalc software 9.0 (MedCalc version 9.2.10).

All data resulted normally distributed based on the Shapiro-Wilk test.

Clinical parameters were evaluated with the One-way ANOVA test to perform a comparison between times. Moreover, histological and immunohistochemical data were analysed with Kruskal-Wallis test followed by Dunn post-hoc test to obtain a comparison between the two groups. All results are presented as mean \pm standard deviation (clinical data) and median (min – max) (histological and immunohistochemical data). Differences with a p value <0.05 were considered statistically significant.

6. RESULTS

6.1 CLINICAL OUTCOMES

No signs of infectious were identified in all subjects.

At T1 and T2, *calor* score was higher than T4 and T5 ($p < 0.05$). Moreover, at T3 it was significantly lower than T1 ($p < 0.05$).

The *rubor* score was significantly lower at T1 and T5 compared to T2 and T3 ($p < 0.05$).

Furthermore, at T2, tumor score was higher than T1, T3 and T4 ($p < 0.05$). Differently, at T5, it was lower than T1, T2 and T3 ($p < 0.05$) (Table 1).

	T1	T2	T3	T4	T5
CALOR	0.27 ± 0.1	0.12 ± 0.05	$0.08 \pm 0.01^*$	$0.04 \pm 0.01^{*\#}$	$0^{*\#}$
RUBOR	0.35 ± 0.18	$0.7 \pm 0.3^*$	$0.78 \pm 0.25^*$	0.58 ± 0.14	$0.38 \pm 0.19^{\# \circ}$
TUMOR	0.73 ± 0.23	$1.35 \pm 0.44^*$	$0.85 \pm 0.3^{\#}$	$0.54 \pm 0.12^{\#}$	$0.38 \pm 0.09^{*\# \circ}$

Table 1. Mean \pm standard deviation of clinical parameters. $^*p < 0.05$ differences compared with T1. $^{\#}p < 0.05$ differences compared with T2. $^{\circ}p < 0.05$ differences compared with T3.

After the first five days, all scores were 0 for the remaining 25 days (Figure 25).

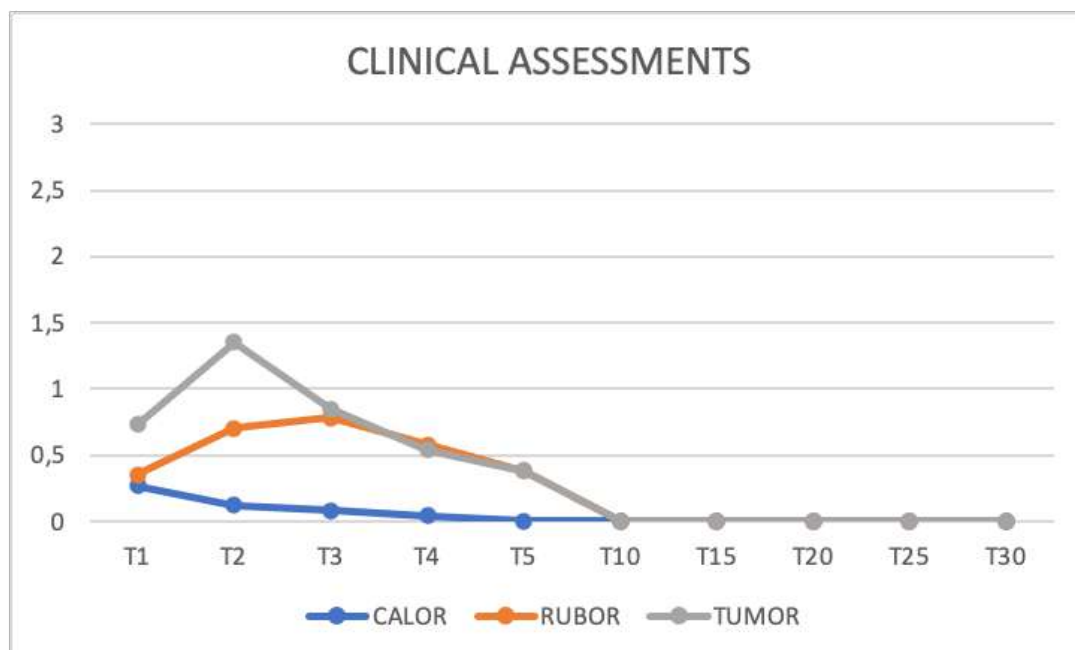


Figure 25. Graphical representation of calor, rubor and tumor scores at the different times of the study.

6.2 HISTOLOGICAL AND IMMUNOISTOCHEMICAL OUTCOMES

Data analyzed showed a mild acute inflammatory response in both groups at the two different times of the study. Specifically, the presence of neutrophils and eosinophiles were no statistical different between A Group and B Group ($p < 0.05$).

Differently, a moderate degree of chronic inflammation was observed. In particular, the lymphocytes cells were higher than macrophages in both groups. Instead, the comparison between two groups showed no statistical differences ($p < 0.05$) (Figure 26).

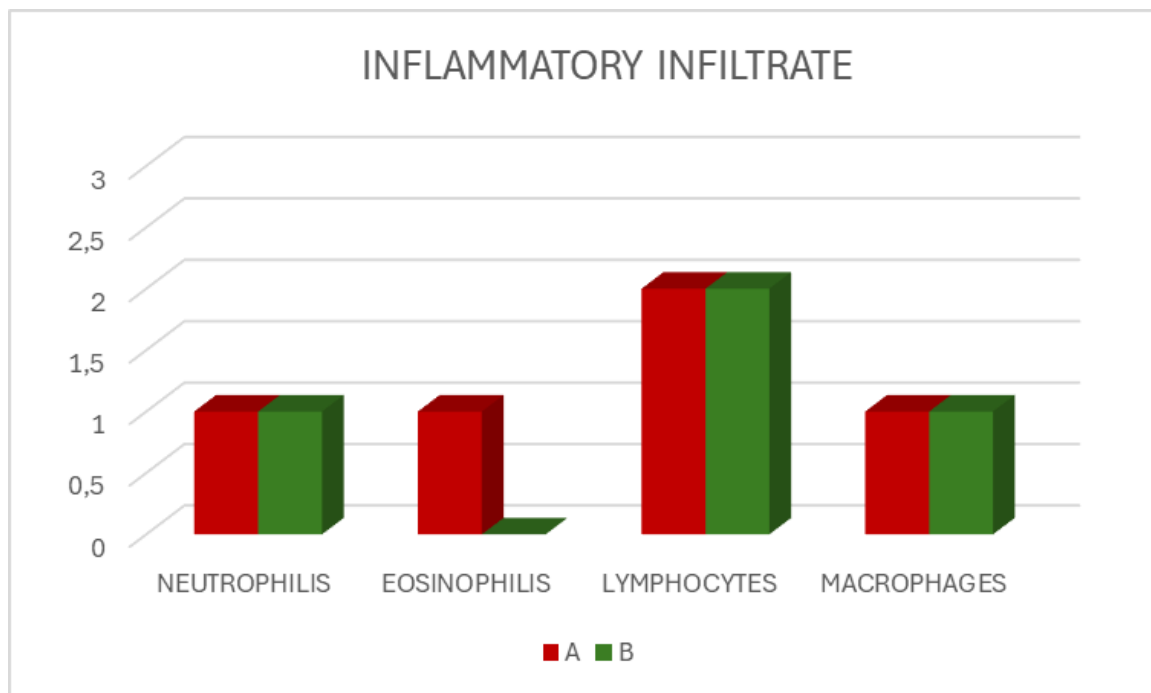


Figure 26. Graphical representation of inflammatory infiltrate scores at the two different times of the study.

The analysis of data related to the scaffold integration showed interesting results. Specifically, neovascularization was significantly higher in A compared to B group. In the same way, cell colonization was statistically major in A group. In contrast, the presence of the capsule was mild in all subjects, and it was no different between two groups of the study (Figure 27; Figure 28; Table 2).

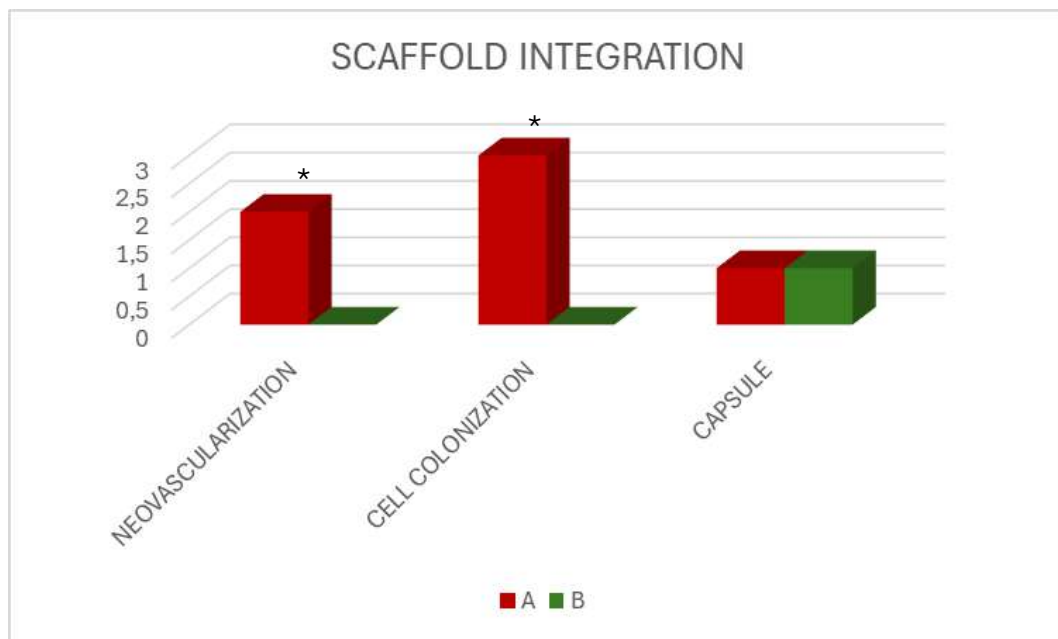


Figure 27. Graphs representation of data related to the scaffold integration at the two different times of the study. * $p < 0.05$ differences compared with B Group.

	A GROUP	B GROUP
NEUTROPHILIS	1 (0-2)	1 (0-2)
EOSINOPHILIS	1 (0-2)	0 (0-1)
LYMPHOCYTES	2 (0-3)	2 (0-3)
MACROPHAGES	1 (0-3)	1 (0-3)
NEOVASCULARIZAZION	2 (1-3)*	0 (0-2)
CELL COLONIZZAZION	3 (1-3)*	0 (0-2)
CAPSULE	1 (0-3)	1 (0-3)

Table 2. Median (min – max) of histological and immunohistochemical parameters. * $p < 0.05$ differences compared with B group.

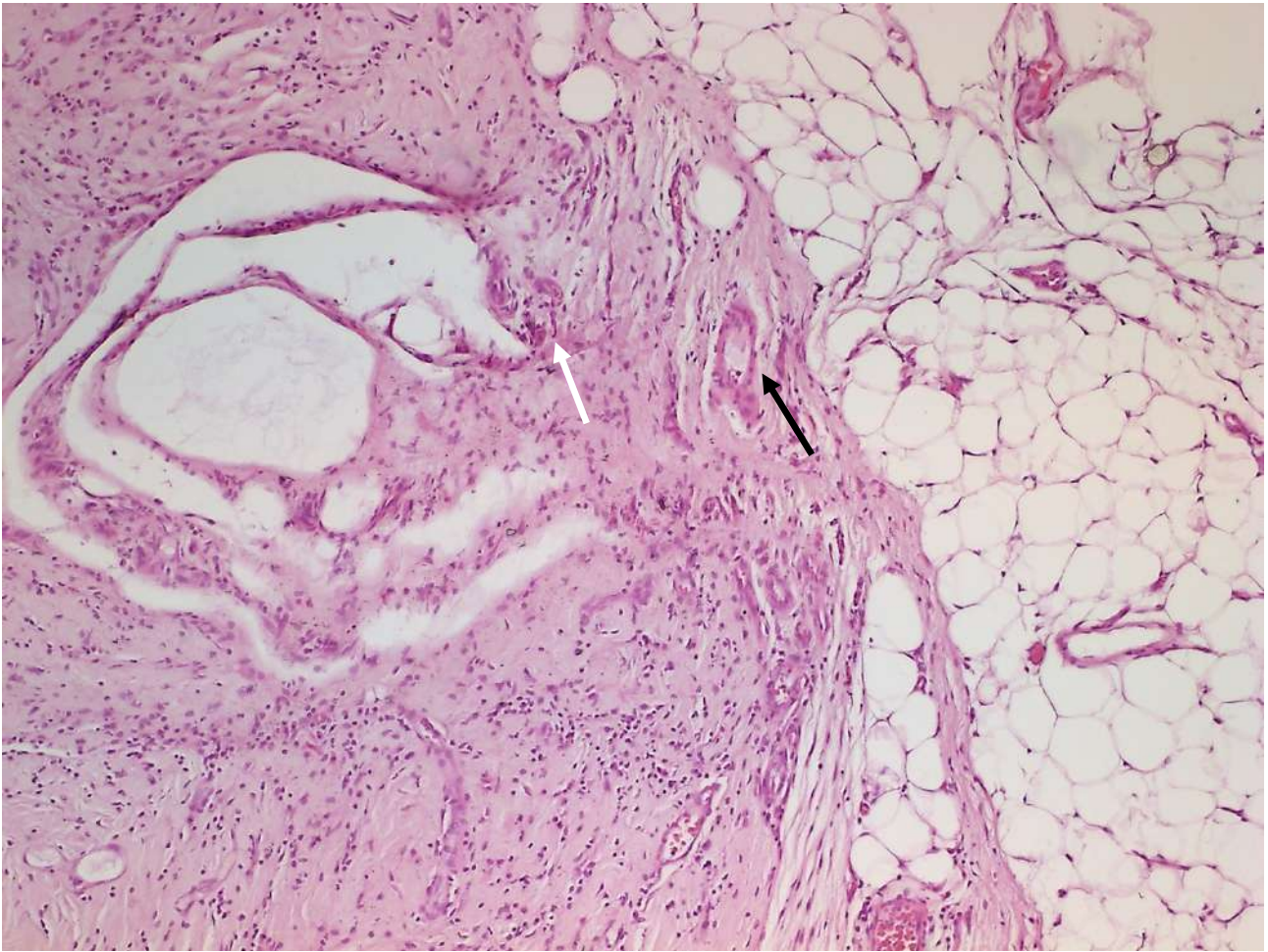


Figure 28. Hematoxylin and eosin at 10 x of the scaffold at the hypodermic level after 30 days. The absence of demarcation between the scaffold and the hypodermis is observed, with abundant cellularization (white arrow) and many microvessels (black arrow). The capsule is not present.

7. DISCUSSION

This is the first study that evaluate the biocompatibility of decellularized extracellular membrane from human foreskin donor in vivo.

Purpura et al. demonstrated in vitro that decellularization of human foreskin with combined physical-enzymatic process eliminates antigenicity and preserves mechanical properties similar to fresh foreskin tissue (Purpura et al., 2018).

The results of our study support that this scaffold has poor immune response and promotes the integration when implanted in hypodermic layer of rat.

The goal of decellularization treatment is to eliminate the immunogenicity of tissue preserving the structural components of extracellular matrix (Jiwangga et al., 2024). However, complete cellular removal is difficult to achieve and a treatment too aggressive could cause inherent damage to extracellular matrix components. Structural damage of ECM elicits foreign body reaction with fibrosis formation and rejection of scaffold (Connor et al., 2009; Zang et al., 2013).

In this study, there were no signs of severe acute inflammatory response. After an acute immune stimulus, neutrophils are the first cells to respond. Subsequently, these are phagocytosed by macrophages (Ji et al., 2021).

The infiltration degree of neutrophils surrounding the scaffold was slight both at 5- and 30-days post implantation. According to Macleod et al., the absence of intense acute inflammation in the first days suggests a degree of tolerance to this scaffold (Macleod et al., 2004).

The poor presence of neutrophils at 30 days was not relate to macrophages phagocytosis because the infiltration of these cells was slight (Ji et al., 2021).

The role of eosinophiles in immune response after decellularized extracellular matrix implantation is lack. It has been reported that the presence of eosinophiles infiltrate may represent a foreign body reaction (Macleod et al., 2004). In contrast, two studies concerning the use of acellular matrix for the treatment of cardiac and nerve injuries showed that the eosinophiles promoted the tissue repair (Vasanthan et al., 2023; Pan et al., 2020). The eosinophilic response may be influenced by the anatomic region, for example, it was stronger in the subcutaneous layer if compared to other space (DeStefano et al., 2023)).

Anyway, a massive eosinophilic infiltration represents severe acute inflammatory response. In our study, the presence of eosinophiles was slight and absent respectively at 5 and 30 days, confirmed a slight acute inflammatory response to the scaffold.

The acute inflammatory response is a physiological event that occurs after a scaffold implantation. The degree and duration are related to the type of scaffold material (Macleod et al., 2004).

Slight acute inflammation is required to start the process of tissue regeneration and normal regeneration of injury tissue needs its control (Suliman et al., 2016).

Subsequently, the chronic inflammatory response arises mediated by macrophages, lymphocytes, and plasma cells. The chronic inflammatory cells may damage the scaffold and elicit a foreign body reaction. However, a minimal chronic reaction surrounding the scaffold support the integration and longevity of this (Macleod et al., 2004).

In our study, we found a slight/moderate chronic inflammation, in particular, the presence of macrophages and lymphocytes were respectively slight and moderate in both groups.

A moderate lymphocytes response may indicate the presence of some residual foreign epitopes in the scaffold (Xu et al., 2010)

Analogous to lymphocytes (Th1 and Th2), the macrophages may present two different phenotypes known as M1 (proinflammatory) and M2 (anti-inflammatory) related to different stimuli. The M1 macrophages are involved in phagocytosis and biodegradation, on the contrary, the M2 macrophages act in tissue repair. In last years, the concept of macrophage polarization has increasingly important in tissue engineering. The switch from proinflammatory to anti-inflammatory macrophages plays a key role for long standing success of the scaffold (Lucke et al., 2015).

In this study, we identified the cells by their morphology alone and did not perform immunohistochemical analysis to evaluate the different types of macrophages.

The immunohistochemical outcomes showed a potential integration of scaffold after 30 days post implantation.

The revascularization is fundamental for tissue integration, however, expedited neoangiogenesis may correlate with increased foreign body reaction (Ji et al., 2021).

We found a moderate neovascularization of scaffold in A group, in contrast, it was absent after 5 days (Menon et al., 2003).

In addition, another key event for integration is the recellularization of scaffold by host. This process begins with inflammatory cells migration and the follows revascularization, that allows recellularization (Capito et al., 2012).

In this study, the fibroblastic colonization of scaffold was massive at 30 days post implantation.

Differently, the presence of fibroblasts surrounding the scaffold were poor. This meant that the fibroblasts of host infiltrated the scaffold and did not form a capsule typically of foreign body. The absence of marked fibrous capsule was recorded in both times of the study (Xu et al., 2010).

In this research, we have to consider also different limits. The first one is related to morphologic identification of inflammatory cells that does not allow to evaluate the different phenotypes with positive or negative effect on scaffold integration.

The second limit is the lack of scaffold degradability assessment. No scaffold degradation at all can prevent cellular activity and colonization (Ji et al., 2021). The stability and clinical efficacy of decellularized extracellular matrix depends on the balance between implant degradation and the neo tissue formation (Zang et al., 2013).

Our macroscopic perception was that the size of scaffold was mildly reduced in the second phase of the study.

The last limit is the relative short time of the study, probably, a more long-term evaluation is needed to highlight a chronic reject.

In conclusion, the results showed that the decellularized extracellular matrix derive from human foreskin did not evoke a significative immune response and supported the neovascularization and cell colonization when implanted in the host.

This scaffold is very promising for reconstruction of human foreskin, however, other studies in animal model are necessary with the purpose of developing a surgical technique to promote the vascularization, innervation and engraftment of a complex structure as human foreskin.

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